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*M. Kaleem Abbasi and M. Nasim Khan*

# Introduction of White Clover for Herbage Production and Nitrogen Fixation in the Hilly Areas of Azad Jammu and Kashmir





*Agricultural sustainability problems related to soil erosion and fertility depletion have arisen throughout the agro-ecological zone of Azad Jammu and Kashmir (AJK). Introduction and exploitation of clover is predicted to be beneficial and useful under these conditions because of its potential for herbage production, $N_2$ fixation, and soil cover characteristics. A 1-year field study with a local ecotype of white clover was carried out. Vegetative growth, herbage yield, nitrogen (N) uptake, and soil N content were investigated. Additionally,* Rhizobia *infestation was studied by isolating* Rhizobia *in culture media. The annual herbage yield (fresh fodder) of clover, and of clover and grass mixture harvested twice during the season was 7–15 t/ha while dry matter production was 3–6 t/ha. Protein content of white clover was 16% as compared to 5% in the indigenous grass species, demonstrating the superior feed value of white clover. The development of* Rhizobium *colonies in the culture media and the presence of about 20 nodules per plant indicated the purity of* Rhizobium *and its $N_2$ fixing potential. The N content of clover and clover and grass mixture was 2–2.5% compared to 0.87% for grass alone, showing that clover was able to fix an average of 77 kg N/ha, a very encouraging figure even under low moisture conditions. A 70% increase in total soil N in clover-growing plots relative to control plots was observed. It was concluded that white clover can easily be grown and established in the hilly areas of Rawalakot, and would sustain a high level of pasture production and provide inputs of N through $N_2$ fixation which, in turn, would increase the productivity potential of soils in mountain eco-regions in AJK.*

**Keywords:** *Herbage yield; soil fertility; pastureland;* Cenchrus ciliaris*;* Rhizobium*;* Trifolium repens *L.; Pakistan.*

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## Introduction

Soil erosion is a major threat to sustainable agriculture in the hilly and mountainous areas of Azad Jammu and Kashmir (AJK), removing the uppermost fertile layer of the soil, depleting fertility, and leaving the soil in poor physical condition. An extensive survey of soils in and around Rawalakot showed a severe deficiency of most major nutrients required for plant growth and crop yield (Malik et al 2000). Among these, nitrogen (N) is extremely deficient in this soil–plant system, the success of which depends entirely upon a continuous supply of this element.

Meeting plant demand for N in a deficient soil is normally achieved by the use of chemical fertilizers, which provide high yields and economic viability for a growing population. But the high cost of mineral N fertilizers and their availability at proper times are the two major constraints responsible for low N fertilizer input. Furthermore, the hilly and sloping landscape of AJK, with high rainfall during both major cropping seasons (*rabi* and *kharif*), can be a cause of inefficient utilization of an expensive input because of surface runoff and leaching losses.

This underscores the importance of developing new production methods that are agronomically and economically sustainable. Introduction and exploitation of legumes under these conditions is the best option because they can grow well alone and in association with grasses and crops over a reasonably wide range of climatic conditions. The supply of year-round and balanced fodder will help in establishing sheep and dairy farms. In addition, the development of legume pastures with perennial grasses will help reduce soil erosion and provide nutritive fodder for animals. Legumes also act as a cover crop, the backbone of any annual cropping system that seeks to be sustainable. The $N_2$ fixing potential of these legumes will further increase N status of the soil and reduce the cost of production by minimizing the use of fertilizers, thereby increasing farmers' incomes.

Pastoral systems in many temperate regions of the world use white clover (*Trifolium repens* L.) because of its feed quality benefits for animals and its inputs of N via fixation of atmospheric $N_2$ (Gibson and Cope 1985; Ledgard and Steele 1992). Although generally regarded as a temperate species found where soil moisture is adequate for growth, white clover is widely adapted to regions from the arctic to the subtropics, and has a wide altitudinal range, reportedly up to 6000 m in the Himalayan region (Sareen 2003). Its phenotypic plasticity and high degree of genetic variability allows white clover to grow and survive in highly variable environments (Woodfield and Caradus 1994).

The widely acknowledged benefits from introducing white clover into the agro-ecosystem are: 1) improvement of soil fertility through atmospheric $N_2$ fixation (Lane et al 2000); 2) forage of high feed value and quality with high crude protein, soluble carbohydrates, minerals such as calcium, magnesium, and phosphorus, and low levels of structural carbohydrates and lignin (Ulyatt et al 1977; Thomson 1984); 3) increased organic matter content and improved soil structure (Mytton et al 1993): white clover is associated with larger rhizosphere microbial

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populations which produce carbohydrate compounds that bind soil particles (Lane et al 2000), reducing soil compaction associated with higher soil porosity and drainage rates (Lane et al 2000); and 4) as a cover crop it reduces erosion and protects soil against degradation (Morgan 1986).

White clover has the potential to fix atmospheric $N_2$ in the range of 600–700 kg/ha/yr N (Crush 1987), although annual $N_2$ fixation levels are extremely variable, ranging from 17 kg/ha/yr N in infertile, unimproved hill pastures (Grant and Lambert 1979) to 380 kg/ha/yr N in intensively managed pastures (Rumball 1979). Although white clover grows naturally all over the hilly areas of AJK, the potential benefits of the indigenous clover species have not yet been explored. One objective of this research was to enhance understanding of the performance of indigenous white clover in terms of its persistence, herbage production, and nitrogen uptake and fixation under the agro-climatic conditions of Rawalakot AJK.

## Study area

The study area (Rawalakot) lies between 1800 and 2000 m, at latitude 33–36° in northeastern Pakistan, under the foothills of the great Himalayas in Rawalakot District, Poonch Division, AJK. The topography is mainly hilly and mountainous, with valleys and stretches of plains. The area is characterized by a temperate sub-humid climate with annual average rainfall ranging from about 500–2000 mm, most of which is irregular, with intense storms during monsoon and winter. The mean annual temperature ranges from a minimum of 0°C to a maximum of 30°C, accompanied by severe cold and snow in winter. Temperature and moisture vary substantially with altitude.

Agriculture is based on a rainfed cropping system and maize (*Zea mays* L.) is the favored crop in the region. Vegetables and fruit trees predominate; the most important fruits are apples (*Pyrus malus*), pears (*Pyrus communis*), apricots (*Prunus armeniaca*), plums (*Prunus domestica*), and walnuts (*Juglans regia*). A large proportion of the area consists of uncultivable wasteland and forest. The important forest species are *deodar* (*Cedrus deodara*), *kail* (*Pinus excelsa*), *cheer* (*Pinus wallichiana*), *sufaida* (*Populus euramericana*), and fir (*Abies pindrow*) (GSJK 1994). The few important grass species found in the area are *Cenchrus ciliaris* (buffel grass), *Digitaria sanguinalis*, *Setaria pallide*, *Poa pratensis*, and *Arundo donax*.

## Materials and methods

### Establishment of clover in the field: nodule collection and isolation of *Rhizobium*

An area of 3x3 m was selected at the research farm, University College of Agriculture, Rawalakot AJK. Soil physical and chemical characteristics are presented in Table 1. Weeds and grasses present in the field were removed manually. The main plot was divided into 12 equal blocks, 2x3 m each. Seeds of indigenous white clover grown naturally were collected and sown at about 5 kg/ha in February 2002. After germination, weeds/grasses were eradicated and only clover was allowed to grow as a monoculture crop.

Before flowering, 4 to 5 healthy and vigorous plants from 6 blocks were carefully uprooted with their full root systems by digging approximately 15 cm to either side of the plant to a depth of at least 20 cm. Each plant was placed in a plastic bucket of water to remove adhering soil particles and care was taken to avoid any damage to nodules. The samples were brought into the laboratory, the tops of the plants were removed, and roots were carefully washed under a gentle stream of water. About 10 healthy, firm unbroken and pink nodules were collected from each plant.

The preparation and isolation of *Rhizobium* were performed by a standard procedure (Subba Rao 1988). The intact nodules were immersed in 95% ethanol for 5–10 seconds in petri dishes, transferred

**TABLE 1** Selected physical and chemical characteristics of the soil used in the experiment.

| Property | Value |
|---|---|
| **Bulk density** | 1.31 Mg/m$^3$ |
| **Particle density** | 2.61 Mg/m$^3$ |
| **Porosity** | 49.8% |
| **Particle size distribution** | |
| **Sand (%)** | 68.0% |
| **Silt (%)** | 19.0% |
| **Clay (%)** | 13.0% |
| **Texture class: sandy loam** | |
| **Calcium carbonate content** | 0.68% |
| **Organic matter** | 0.90% |
| **pH (1:1 water)** | 7.8 |
| **Total N** | 760 mg/kg |
| **Available N ($NO_3$-N)** | 1.88 mg/kg |
| **Available phosphorus** | 2.5 mg/kg |
| **Available potassium** | 83.0 mg/kg |

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immediately to a 3% solution of hydrogen peroxide ($H_2O_2$), and soaked for 4–5 minutes to surface sterilize the nodules in small beakers. Immediately following sterilization, nodules were rinsed in 5 to 7 changes of sterile water using sterile forceps for transferring. They were then dipped into ethyl alcohol, followed by more washing with sterile water. Sterilized nodules were crushed in petri dishes with a sterile glass rod, using 1–2 drops of sterile water to make slurry. One loop full of nodule suspension was streaked on the surface of Yeast Extract Mannitol Agar (YEMA) plates/test tubes with the help of a smooth glass rod. A total of 24 test tubes were prepared accordingly. The plates/test tubes were incubated up to 10 days in the incubator at 25±2°C. When the large gummy colonies of bacteria emerged within 5–7 days, the *Rhizobium* colonies (isolates) were identified for purification. The inoculants were stored at 25–30°C. Daily observations were made for the appearance of colonies typical of *Rhizobium*.

## Growth characteristics, herbage yield and $N_2$ fixation

The growth parameters, ie plant height and root length, were recorded by selecting 4 plants randomly from each of 6 plots, when the plants attained almost 50% flowering. For the sampling of roots, plants were uprooted in accordance with a method adopted for nodule collection. The same plants were used for height measurement. A full plant was tied up with a long stick and measurements were recorded with a ruler.

The first cutting of herbage from each of 6 plots was carried out during the second week of May 2002. Samples were weighed to determine herbage fresh weight (FW) and dried in Cryovac bags for 48 hours at 70°C for their dry weight (DW). In the second phase of the experiment, the indigenous grass species (*Cenchrus ciliaris*), which was not allowed to grow and eradicated during the first phase of the experiment, was allowed to grow this time in the clover plots till the final harvest. In the second week of August, grass and clover sward was cut out from each plot for its fresh and dry weight, as described above.

A part of the dried herbage samples was milled and sieved to determine total N. The amount of $N_2$ fixed by an individual legume plant that is entirely dependent on fixation can be measured simply by analyzing the whole plant for total N. The method used in the present investigation is the difference in total N between fixing and non-fixing plants, as described by Whitehead (1995). N uptake by plants, both in the first and second harvests, was determined by the Kjeldahl method (Bremner and Mulvaney 1982).

### Soil analysis

After the final harvest, soil samples were taken from 3 points within each of 6 plots. Samples were taken from depths of 0–20 cm. Samples from each point in the plot were bulked and mixed for subsequent analysis. Similarly, soil samples from fallow plots without clover were also collected accordingly. Total N in soil was determined following the method of Bremner and Mulvaney (1982). Comparisons were made between the 2 soils.

## Results and discussion

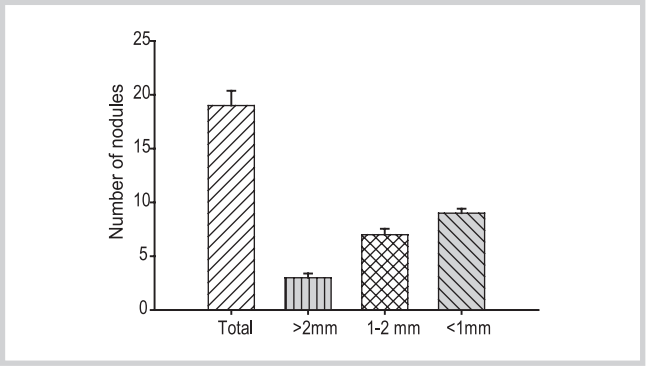
### Isolation of *Rhizobium* and nodulation of white clover

Collection of nodules is generally performed to supplement a strain collection with superior strains (Beck et al 1993). In the present study, daily observations were made for the appearance of colonies typical of *Rhizobia*. The samples started producing colonies after day 5 and the maximum appearance was found on day 7 (data not shown). A visible and significant appearance of colonies in the culture media was observed, indicating the presence and purification of *Rhizobium* capable of forming a symbiotic relationship with the host (clover) and fixing atmospheric $N_2$. The number of nodules varied from 16–24, with an average of 19 nodules per plant (Figure 1). This substantial number of nodules exhibited the potential of $N_2$ fixation. The size of root nodules varied, with a maximum of 47% nodules below 1 mm diameter followed by 37% nodules of 1.5 mm in diameter. Although the ratio between total root weight and number of nodules was not compared, it has been shown that in total the root nodules may comprise up to 20–35% of the total root weight when the plant is relying entirely on fixation for its N supply (Ryle et al 1979; Arnott 1984).

### Vegetative growth and herbage yield

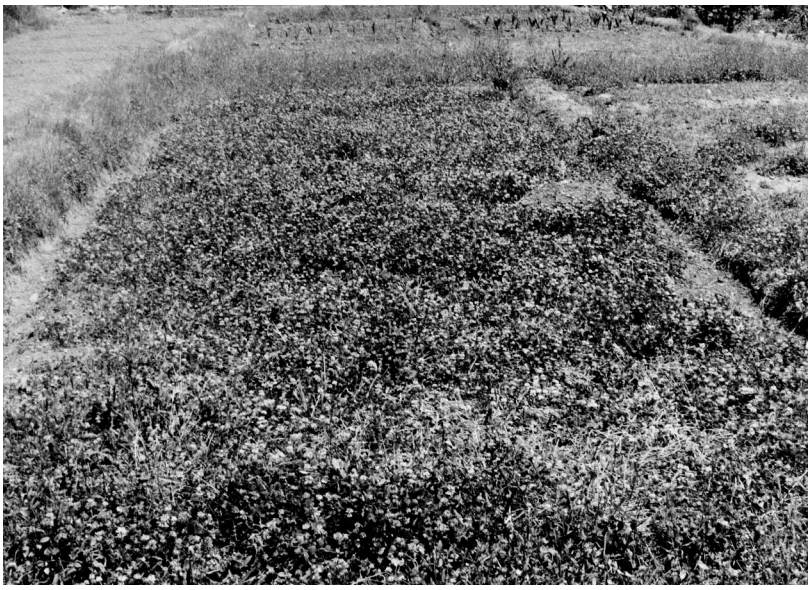
A general view of field plots of white clover grown under Rawalakot conditions is shown in Figure 2. The

**FIGURE 1** Number and size of nodules collected from indigenous white clover grown in Rawalakot District, Azad Jammu and Kashmir. The vertical lines on the bar represent the standard error of means (SEM)(n=6).




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**FIGURE 2** General view of field plots of white clover grown at the Research Farm, University of Azad Jammu and Kashmir, Faculty of Agriculture, Rawalakot. (Photo by authors)



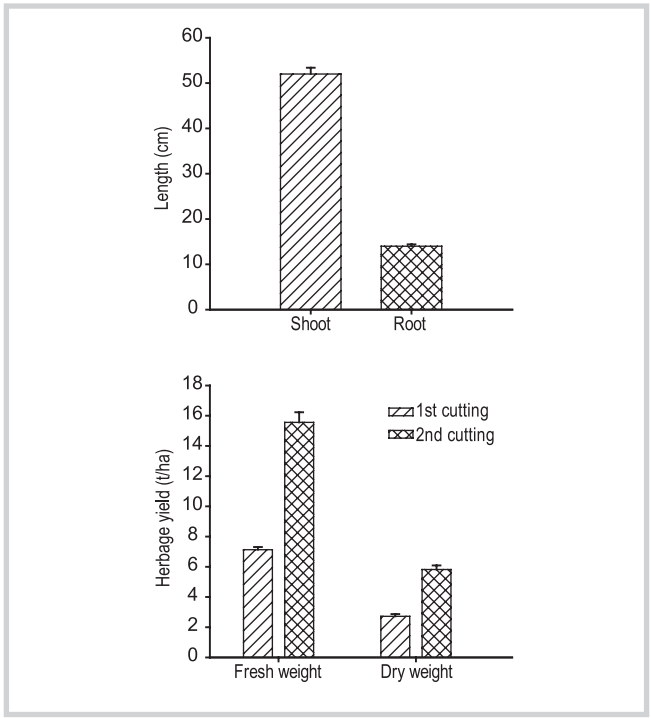
plants appeared healthy and showed considerable growth during the season. Plant height varied from 44–60 cm (Figure 3), indicating white clover's potential for growth and establishment under Rawalakot conditions. Any pasture of this height can easily be used as a source of fodder, green manure, etc. In the first harvest, a 6–8 t/ha of fresh fodder yield was recorded, while the dry matter yield ranged from 2.0–3.5 t/ha (Figure 3), which was 3 times that of grass grown as a monoculture.

Germination of clover started at the end of March and continued till mid April, and the crop was harvested (first cut) in the middle of May. For 1½ months, more than 7 t/ha (average) of fresh fodder was produced, which is a substantial quantity in fodder-scarce areas such as Rawalakot AJK. In the second harvest, the fresh fodder yield was 16 t/ha (average), while the DM yield was 5.8 t/ha, indicating almost a double yield when clover and grass were grown as a mixture (second cut) compared to clover monoculture (first cut; Figure 3). From both cuts, the total above-ground DM yield was 8.2 t/ha, which was almost identical to that reported by Hoyt (1987) for crimson clover. Similarly, Whitehead (1995) reported a 2–4 t/ha yield of DM for white clover, depending mainly on soil moisture. Elgersma and Hassink (1997) reported that the DM yield of white clover ranged from 2.4–11.2 t/ha, while the grass monoculture produced 2.1 t/ha DM.

Generally, 3–5 cuts of white clover per season were reported in the areas with improved clover cultivars, and clover remains active as a perennial crop throughout the year. However, in the present study only 2 cuts were taken because low moisture supply in summer and low temperature in winter restricted clover growth and persistence. This is a common problem with indigenous clover species, and increasing winter activity and tolerance to summer moisture stress is a common breeding objective for many countries. Introduction of cultivars with higher productivity under conditions of summer moisture stress and cold temperatures in winter in countries such as Australia, where these are important breeding objectives, may alleviate this problem.

In addition, the phosphorus (P) level of the soil used in the study was 2.5 mg/kg (Table 1), a concentration typical of the soils in the hilly regions of AJK (Malik et al 2000). This would normally be regarded as a very low level for species such as white clover, which demands a high level of phosphorus fertility (Hogh-Jensen et al 2002) and could be a possible factor limiting white clover growth. There is evidence to suggest that tolerance to moisture stress in white clover is higher when P nutrition is adequate (Singh and Sale 1998; Singh and Sale 2000; Singh et al 2000). Therefore, P has an influence both on stress tolerance and herbage yield, and could be considered a limiting factor in the present investigation.

**FIGURE 3** Length of shoot and root (cm), and fresh and dry matter yield (t/ha) of white clover (average from 6 plots). Vertical lines on each bar represent the SEM (n=6).




## Herbage analysis: N uptake and fixation

The N contents of white clover were 2.24–2.67%, while the N in grass (reference plant) was 0.76–0.95% (first cut), indicating that clover was able to assimilate 3 times more N than grass under similar soil and environmental conditions (Figure 4). This additional N

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**FIGURE 4** N content (uptake of N) in percent in the 1st and 2nd cuttings of clover and grass, and $N_2$ fixation by white clover grown as monoculture (left) and mixed with indigenous grass sward (right). Vertical lines on each bar represent the SEM (n=6).

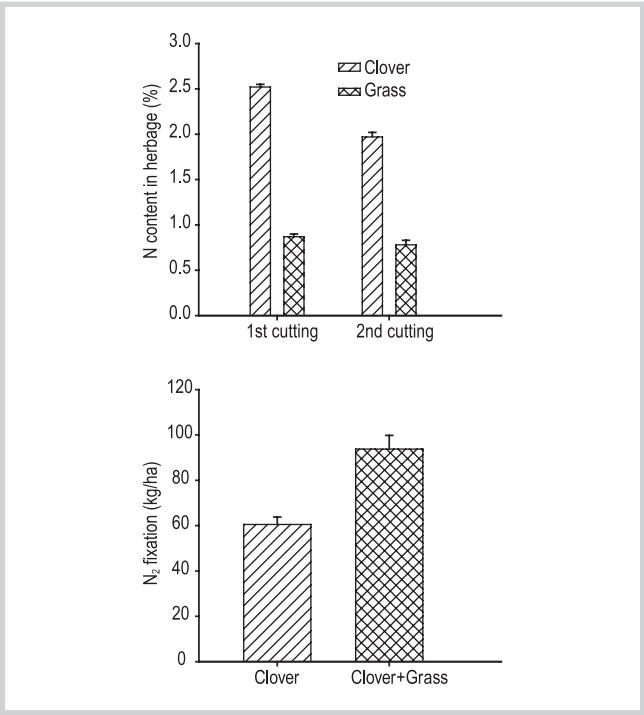
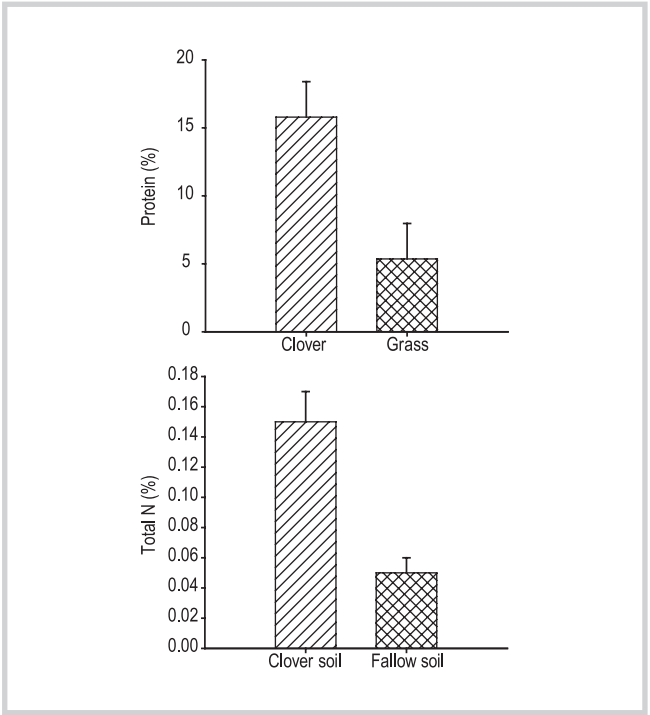



**FIGURE 5** Protein content (%) of white clover and indigenous grass species, and total N (%) in clover and fallow soil determined after crop harvest. Vertical lines on each bar represent the SEM (n=6).




was most likely due to $N_2$ fixation, as white clover was able to fix 45–86 kg/ha, with an average of 61 kg/ha N (Figure 4). In the second harvest (grass and clover), the uptake of N varied from 1.76–2.22% (Figure 4),while the amount of N fixed by grass and clover swards varied from 51–147 kg/ha N, with an average value of 94 kg/ha N. By taking the average of both the cuttings, 77 kg/ha N was fixed by white clover during the whole season (Figure 4). Abberton et al (1999) indicated 3.2–4.4% of N content in white clover, while Simpson et al (1988) reported a range between 3.5–5.8%. However, defoliation may affect N uptake, and the turnover may amount to about 2% (Marriott and Haystead 1990).

In the present study, relatively low N uptake was attained; proper management practices and use of phosphorus as an additional nutrient might increase N efficiency and recovery in clover. Annual $N_2$ fixation levels from white clover are reported as extremely variable, ranging from 17 kg/ha/yr N in infertile, unimproved hill pastures (Grant and Lambert 1979) to 380 kg/ha/yr N in intensively managed pastures (Rumball 1979). Lane et al (2000) reported that the rate of $N_2$ fixation by white clover varies greatly, strongly influenced by clover vigor and moisture supply. Nitrogen fixation for developed pastures in New Zealand has been estimated between 100 and 300 kg/ha/yr N (Brock et al 1989). Fixation rates of 138–212 kg/ha/yr N were measured in Tasmania, Australia (Lane 1985). The amount of $N_2$ fixed in the present study was relatively low compared to amounts reported earlier, probably because *Rhizobium* was unable to utilize its full extent to capture atmospheric $N_2$ and assimilate it into the plant body. This is attributed to extreme drought after mid May, which existed throughout the country after 1997, decimating livestock and severely affecting horticulture and rainfed agriculture.

According to a report by the Food and Agriculture Organization (FAO) and the World Food Program (FAO 2001), rainfall was between 50–80% below normal in most parts of the country during 2001. These conditions did not change much in 2002. However, the hilly areas of AJK basically have summer agriculture, as there is generally enough rainfall in summer, except in the past few years.

The effect of drought on $N_2$ fixation in white clover was also described by Crouchley (1979), who reported that $N_2$ fixation amounted to 90 kg/ha N in a year with a dry summer, but 240 kg/ha N were obtained in the following year with a moist summer. Moisture stress is a major limiting factor for white clover, and its growth decreased rapidly at soil moisture levels below 35 mm or 60% of relative soil moisture (Lane et al 2000). It was predicted that a minimum of 700 mm average annual rainfall is required for significant presence of white clover in pasture (Hill et al 1996). However, even under unfavorable climatic conditions, fixation of 61 kg/ha N in the first cut and 94 kg/ha N in the second cut is a

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substantial amount in a nutrient-scarce environment. The proper utilization of clover with this potential in the agro-ecosystem could save 77 kg/ha N otherwise needed from fertilizers.

### Soil analysis total N

The soil N in plots under clover ranged from 0.094–0.213% (0.154% average), while the fallow plots had relatively less N, ranging from 0.028–0.059% (Figure 5). Results indicated that the plots with clover had almost 70% more N than control plots. It is likely that clover enriched the soil with N through $N_2$ fixation.

The quality of white clover as a fodder seemed excellent, as the overall protein content of white clover was 16% relative to 5.5% in grass, ie the reference plant (Figure 5). White clover also acts as a cover crop, which could be considered the backbone of any annual cropping system that seeks to be sustainable. Figure 6 shows the clover plant's width, attachment, and creeping potential to the soil surface. This potential of clover can be further exploited to combat soil degradation, especially in reducing erosion, which is a major problem of soil and crop productivity in the hilly and sloping areas of AJK.

**FIGURE 6** View of a white clover plant, showing N-fixing roots and leafy abundance of clover as a cover and fodder crop. (Photo by authors)



## Conclusions

This experimental study focused on the establishment of white clover and its persistence, isolation of *Rhizobium*, and the potential of clover for herbage production and $N_2$ fixation. The results were very encouraging, showing that substantial herbage production of clover contributed 7–15 t/ha of fresh fodder yield. The introduction of white clover with indigenous grass species doubled the yield relative to white clover monoculture, and this mixed sward can be used for fodder conservation in winter. The morphological features of the plant showed white clover to be an excellent source of fodder for local use, having very high protein content (16%). The $N_2$ fixing potential of the crop is also substantial, and clover and grass swards were able to fix 77 kg/ha N. The incorporation of clover in soil may play an important role in the N benefits to subsequent crops, and/or contribute to increased soil N fertility. Results of this study suggest that white clover has a tremendous potential for growth, both as a fodder and an $N_2$ fixing species also capable of enriching the soil with N. The introduction and exploitation of indigenous as well as imported white clover varieties in our agro-ecosystem can supply a year-round, balanced type of fodder for animal production, improve soil health and soil quality, and increase the N status of soil. The effect of white clover on the physical conditions of soil and as a cover crop, as well as its effect on soil erosion, should also be included in future research.

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